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14. ABSTRACT

Gliomas comprise ~60% of cases among pediatric brain tumors. To date, there are few resources available to study and manipulate pediatric brain tumor cells, to evaluate whether pediatric tumor will have fundamental different responses to the new therapeutic regimes. Since glioma stem cell lines have been successfully isolated from adults, in this proposal we aim to isolate and characterize GSC populations from pediatric patients. In the past two years we have successfully derived and cultured eight patient-derived pediatric glioma stem cell lines. In the past year we have continued molecular and phenotypic characterization of these lines. This characterization included analysis of gene expression and patient-specific gene mutations, and also proof-of-concept shRNA screens. In addition we have begun to identify candidate therapeutic targets using the pediatric GBM isolates. So far, this effort has led to two promising candidate molecules for precision pediatric brain tumor therapeutics.

15. SUBJECT TERMS
Glioblastoma multiforme , glioma stem cell, brain tumor-initiating cell, pediatric brain tumor, RNAi, functional genetics

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Title: Pediatric Glioblastoma therapies based on patient-derived stem cell resources
Contract Number: W81XWH-11-1-0756

PI: Patrick J. Paddison, PhD, Fred Hutchinson Cancer Research Center
Progress Report September 2013 (YEAR 2)

INTRODUCTION

Glioblastoma multiforme (GBM) is the most aggressive and common form of brain cancer in adults, and is among the deadliest cancers with a median survival period of 12-14 months. GBM tumors appear to be hierarchically organized suggestive of a cancer stem cell origin. Consistent with this notion, tumor-initiating, glioma stem cells (GCS) have recently been isolated that retain the development potential and specific genetic alterations found in the patient's tumor. When used to generate tumors in the cortex of mice, these cells give rise to patient-specific molecular signatures and histological features.
Gliomas comprise ~60% of cases among pediatric brain tumors. To date, there are few resources available to study and manipulate pediatric brain tumor cells, to evaluate whether pediatric tumors will have fundamental different responses to the new therapeutic regimes. Since glioma stem cell lines have been successfully isolated from adults, in this proposal we aim to isolate GSC population from pediatric patients. Through collaboration with Dr. Xiao-Nan Li at Texas Children's Hospital, Dr. Paddison's group has access to ten orthotopic mouse lines harboring tumors derived from ten different pediatric glioma cases. In these orthotopic xenograft models, patient tumor samples are injected directly into the cortex of recipient NOD-SCID mice, where upon the patient tumor regrows with similar molecular and pathological characteristics observed in the tumor of origin. In this grant, we are deriving and characterizing pediatric GSC lines and assessing whether they diverge from adult GSCs with respect to genes and networks required for proliferation and survival.

BODY

In this proposal, Dr. Paddison takes a step towards defining new therapeutic strategies for pediatric glioma by applying adult GSC isolation and culture techniques to derive pediatric glioma stem cells and then assessing whether pediatric isolates diverge from adult GBM patients with respect to genes and networks required for proliferation and survival. There are three specific aims:

1. To isolate pediatric glioma stem cell populations in defined monolayer growth culture conditions.
2. To perform molecular and phenotypic characterization of pediatric glioma stem cell isolates.
3. To determine whether adult and pediatric GSCs share common proliferation and survival networks.

During year 2, excellent progress has been made towards the goals of this grant. However, while the overall statement of work has not changed in year two, some aspects of the work plan have progressed slower, while others have progressed faster, as is the norm with biological experiments, especially with patient cancer isolates. **Thus, we present progress towards both year 2 and year 3 goals below that occurred during year 2 (with Aim 1 having been completed)**

Progress on Aim 2

The overall goal of this grant is to create tractable pediatric glioma tumor models and to use them to identify new therapeutic approaches to treat this terrible disease. During year 2, we have been able to further characterize 3 of the 8 pediatric patient lines derived in year 1, including 1502 (GBM; 4y8mo / female), 1406 (GBM; 4y / female), and 3752 (GBM; 5y / female). While each of the eight isolates can grow as neurospheres (Year 1 Progress report Table 1), only these three isolates can be converted to robust monolayer cultures and grown in a tractable manner with doubling times <60 hrs, similar to our adult

GSCs. As a result, we have chosen to focus on these for further inclusion in molecular characterization and functional genetic experiments in Aims 2 and 3.

In aim 2, we proposed to perform molecular and phenotypic characterization of pediatric isolates from Aim 1 to determine whether they: (1) harbor glioma stem cell characteristics; (2) fall into one or more adult GBM subclasses; and (3) are capable of initiating tumors. We have made significant progress for this aim.

In year 1 we began to assess pediatric isolates in terms of their ability to form tube-like endothelial structures in vitro, which may be important for tumor-microenvironment interactions and represent a novel tumor-specific lineage [6,7]. So far the 1406 but not the 1502 line shows ability to differentiate and form tube-like structures in endothelial tube formation assays. We've also started access degree of expression of endothelial cell markers such as CD105, which may correlate with tumor aggressiveness in adult GBMs. In year 2, we extended this analysis to include differentiation conditions that promote GBM-derived pericytes [1] that interact with endothelial cells and may be a key feature of GBM differentiation programs. Through a collaboration with Dr. Shideng Bao (Cleveland Clinic) we have begun to assess the ability of the pediatric glioma cell lines to convert into pericytes using Dr. Bao's fluorescent reporter systems [1] and have confirmed expression of pericyte-specific genes in pediatric glioma stem cell lines. These results suggest that similar to adult brain tumors pediatric ones have the ability to form pericytes (**Figure 1**).

In year 1 we were in the process of classifying pediatric GSC isolates according to the scheme proposed for adult GBMs [8,9] based on an 840 gene list predictive of Proneural, Neural, Classical and Mesenchymal subtypes. In year 2 we've completed refining the method for determine subtype and have applied it to pediatric isolates 1406 and 1502 (**Figure 2**). The method we developed and refined (yr 1-2) is as follows. We performed RNA-seq on GSC cultures (n=3) using an Illumina HiSeq 2000 according to the manufacturer's instructions (FHCRC Genomics Shared Resource). RNA-Seq reads are then aligned to the GRCh37/hg19 assembly using Tophat [11] and counted for gene associations against the UCSC genes database with HTSeq, a python package for analysis of high-throughput sequencing data [12]. The R language of statistical computing is then used for further analysis [13]. All data is combined and normalized using a trimmed mean of M-values (TMM) method from the R package, edgeR [14-16]. Normalized counts are then log transformed, and the means across all the cell lines were used to calculate relative gene expression levels. The GSC line data is then clustered using a Manhattan distance complete-linkage method to establish leaflets. Previously 173 glioma cell lines were subtyped using the expression of 840 signature genes [8]. Our samples are clustered using 790 of these genes. The associations of our cell lines to those in publication are determined by minimum Manhattan distance to expression centroids produced by ClaNC. If a gene is expressed consistently in a particular subtype by absolute distance, then that is counted as a 1 and the number of associated genes in each category is summed. As a validation, the four subtypes are clearly distinguished when the method is applied to the 173 glioma lines described previously [8]. Using this analysis 1406 is "proneural" and 1502 is "mesenchymal" (**Figure 2**).

In the past year, we have also performed exome-sequing on 1502 and potentially discovered novel mutation spectra different than that for adult glioma isolates. We have now started the process of building significant work flows (in collaboration with Dr. Jun Zhu (MSSM)) to weed out common single-nucleotide polymorphisms and also to using RNA-seq data to predict copy number variation from RNA-seq data of the pediatric lines.

Progress on Aim 3

In aim 3, we proposed to examine RNAi hits that have been validated as essential for adult GSC proliferation or survival in pediatric GSC isolates. In addition, we further proposed to perform preliminary shRNA "shot gun" screens to be performed on two pediatric GSC lines to obtain functional genetic "finger prints" to compare to adult GSCs screen results. Last year we reported testing several candidate glioma-lethal targets in pediatric glioma isolates, including two that have been recently published BUB1B [17] and PHF5A [18]. Of these we found that pediatric isolates had variation in requirement for BUB1B but not for PHF5A. Using RNA-seq and exome-seq data for our isolates, in collaboration with Jun Zhu (MSSM, NY),

we have discovered both gene expression and mutation signatures that may correlate with BUB1B sensitivity. If successful, we may be able to predict sensitivity to BUB1B inhibition based on molecular signature of a handful of genes (e.g., 1-10) – hopefully in year 3 we will have further answers.

In addition, from the results published in [18] we have found a novel vulnerability in ZNF131 for pediatric isolate 1502 (**Figure 3**). According to motif analysis ZNF131 encodes a BTB/POZ zinc finger protein, and thus might act as a transcriptional regulator. We are currently pursuing the hypothesis that ZNF131 transcriptionally regulates HAUS5, a component of Augmin protein complex, and that this regulation is crucial for maintenance of 1502 and other tumor isolate viability. In year 3 we will present additional data regarding this hypothesis and biology.

In addition have also performed RNAi screens targeting 319 epigenetic factors in adult and pediatric glioma isolates and also in neural stem cells, a non-transformed candidate cell of origin control for gliomas [17,18]. Our preliminary results from this screen are presented in **Table 1**. The goal of this screen is to identify key epigenetic factors that are required for maintenance of expression of survival and self-renewal circuits in adult and pediatric glioma stem cells. However, this functional set has the added advantage of targeting genes with drugable enzymatic activities. As a result we are already collaborating with Cheryl Arrowsmith of the Structural Genomics Consortium at University of Toronto, who has helped develop cell permeable inhibitors of the Set1-like multiprotein histone methyltransferase complex, two members of which scored in our screen. However, we have noticed some noise in our data analysis and may end up having to redo this screen in year 3 to ensure that hits our valid. We have also acquired an improved RNAi library, which will help facilitate this process (~10 shRNA per gene rather than only 3 for our current one).

KEY RESEARCH ACCOMPLISHMENTS during year 2

- **Isolation and propagation of tumor initiating cells in monolayer culture from 8 pediatric glioma patients**
- **Development and use of methodology for classification of GBM tumors from RNA-seq data**
- **In depth mutation scan and RNA-seq comparisons for 1502 glioma isolate and development of genomics analysis pipeline**
- **Confirmation of GBM-lethal candidates from adult GBM isolate screens in a pediatric GBM isolate**
- **RNAi screen 1502 GSC line for key epigenetic factors required for pediatric glioma stem cell self-renewal**

REPORTABLE OUTCOMES

- manuscripts, abstracts, presentations:
  Ding Y et al., Cancer-specific requirement for BUB1B/BUBR1 in human brain tumor isolates and genetically transformed cells**. Cancer Discov**. 3(2):198-211, 2013

  Hubert CG et al., Genome-wide RNAi screens in human brain tumor isolates reveal a novel viability requirement for PHF5A. **Genes and Dev.** 27(9):1032-45, 2013

- licenses applied for and/or issued: none during this reporting period
- degrees obtained that are supported by this award: none during this reporting period
- development of cell lines, tissue or serum repositories:
  Isolation and propagation of tumor initiating cells from eight pediatric brain tumor patients
- infomatics such as databases and animal models, etc.:
  Development of methodology for classification of GBM tumors from RNA-seq data and exome sequencing and shRNA screening.
- funding applied for based on work supported by this award: **Pardee Foundation and NIH R01.**

- employment or research opportunities applied for and/or received based on experience/training supported by this award: none during this reporting period

CONCLUSION

In summary, we have made substantial progress towards developing *in vitro* cell models for pediatric brain tumors to identify new therapeutic targets for pediatric glioma for Aims 2 and 3, despite having a few set backs with regard to growth of certain isolates in monolayer culture. However, if progress continues to be made in year 3, we will have at least >3 strong lead candidates for translation into therapeutic development pipelines for treating pediatric glioma as a result of using the pediatric resources developed in this grant.

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SUPPORTING DATA

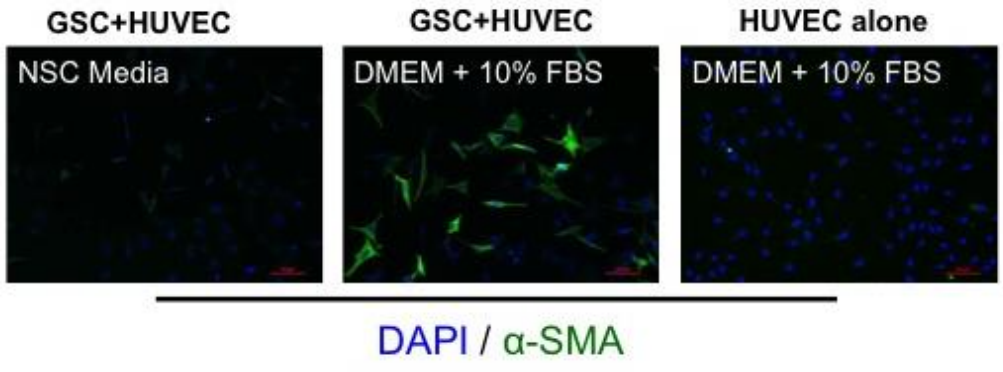

Figure 1. Pericyte differentiation of GSCs co-cultured with human endothelial cells (HUVECs). A recent study demonstrated that GSCs have the capacity to form functioning pericytes, a cell type important for blood vessel generation, structure, and function, as well as for maintenance of the blood-brain barrier maintenance [1] . A 1:1 admixture of GSCs and HUVECs (or HUVECs alone, right panel) was seeded onto laminin coated dishes followed by treatment normal NSC expansion media (left panel) or DMEM + 10% FBS for seven days. Afterwards, cells were fixed and stained for DNA content (DAPI) and alpha smooth muscle actin (α-SMA), a key marker of pericytes which is not expressed in self-renewing GSCs or normally dividing HUVECs. The middle panel shows dramatic induction of α-SMA in GSCs co-cultured with HUVECs. This will be used as a phenotype assay in Aim 2 for our panel of pediatric GSCs isolated from Aim 1.

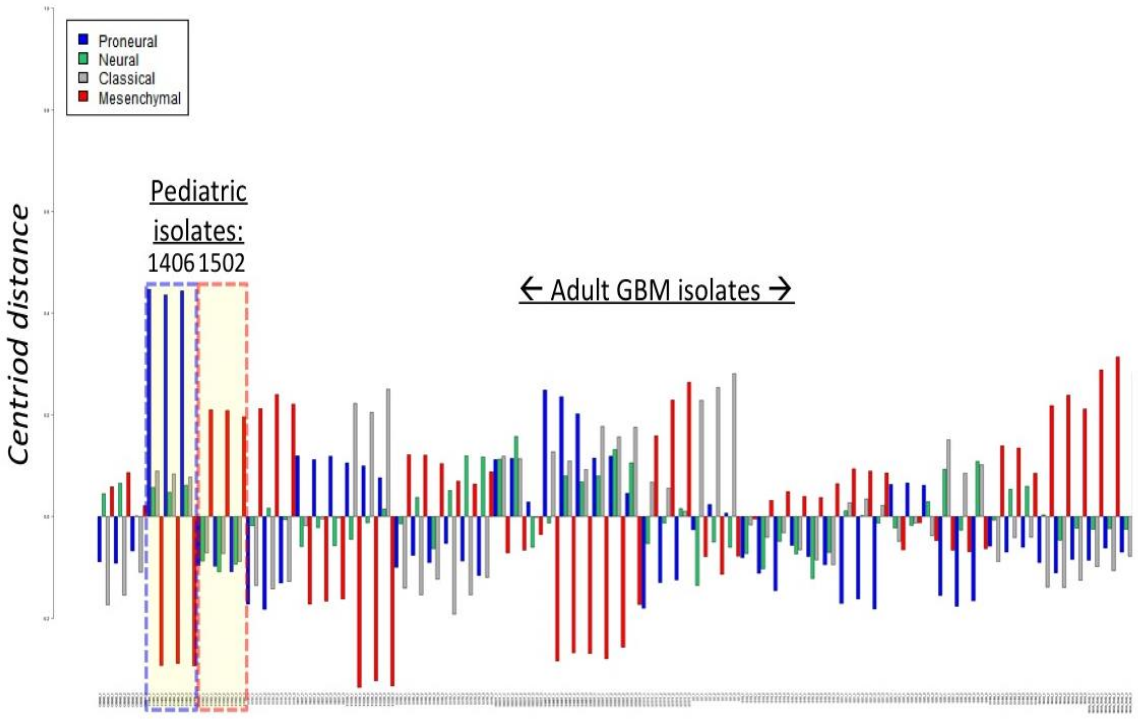

Figure 2. Glioma developmental subtype analysis of pediatric and adult glioma isolates (see text for details). Results suggest that 1406 is "proneural" and 1502 is "mesenchymal".

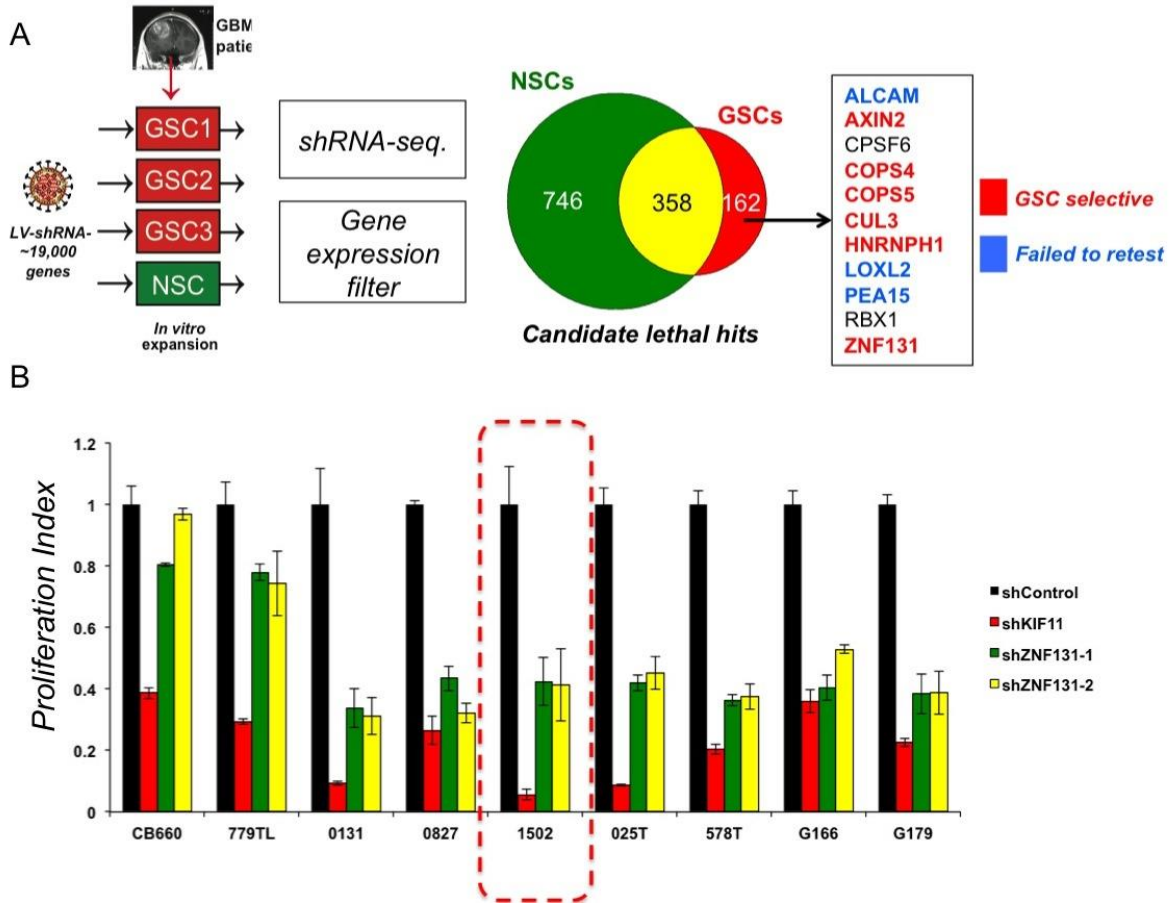

Figure 3. Genome-wide screens from [18] in patient-derived GBM stem-like cells and control neural stem cells revealed an novel candidate ZNF131 (A). ZNF131 was further validated in outgrowth assays for viability using both adult and pediatric GSCs (B). ZNF131 showed specific lethality in tumor isolates. Pediatric isolate 1502 is highlighted in red-hashes. Note that KIF11 knockdown acts as an essential gene control and the CB660 and 779TL are both human fetal neural stem cell isolates used as candidate cell-of-origin controls for GSCs.

Table 1: Results from a shRNA screen comparing adult and pediatric GSCs to NSCs, attempting to identify new therapeutic targets from among 319 epigenetic regulatory factors.

Gene	Predicted Function	shRNAs scoring	Specificity?
ASH2L	Set1-like multiprotein histone methyltransferase complex	3	Adult/Pediatric
DPY30	Set1-like multiprotein histone methyltransferase complex	4	Pediatric
KAT5	Histone acetyltransferase	3	Pediatric
KAT8	Histone acetyltransferase H4K16ac	4	Pediatric
PARP2	Poly ADP-ribosyl transferase-like 2 protein	3	Pediatric
PBRM1	Protein polybromo-1	4	Pediatric
PRDM11	PR Domain-Containing Protein 11	4	Pediatric
SIN3B	Transcriptional repressor	4	Pediatric
SIRT7	NAD^+-dependent deacetylase	4	Adult/Pediatric
USP22	Deubiquitinating enzyme	4	Pediatric